

Trapping a translocating protein within the anthrax toxin channel: implications for the secondary structure of permeating proteins

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Anthrax toxin consists of three proteins: lethal factor (LF), edema factor (EF), and protective antigen (PA). This last forms a heptameric channel, (PA₆₃)₇, in the host cell's endosomal membrane, allowing the former two (which are enzymes) to be translocated into the cytosol. (PA₆₃)₇ incorporated into planar bilayer membranes forms a channel that translocates LF and EF, with the N terminus leading the way. The channel is mushroom-shaped with a cap containing the binding sites for EF and LF, and an ~100 Å-long, 15 Å-wide stem. For proteins to pass through the stem they clearly must unfold, but is secondary structure preserved? To answer this question, we developed a method of trapping the polypeptide chain of a translocating protein within the channel and determined the minimum number of residues that could traverse it. We attached a biotin to the N terminus of LF_N (the 263-residue N-terminal portion of LF) and a molecular stopper elsewhere. If the distance from the N terminus to the stopper was long enough to traverse the channel, streptavidin added to the trans side bound the N-terminal biotin, trapping the protein within the channel; if this distance was not long enough, streptavidin did not bind the N-terminal biotin and the protein was not trapped. The trapping rate was dependent on the driving force (voltage), the length of time it was applied, and the number of residues between the N terminus and the stopper. By varying the position of the stopper, we determined the minimum number of residues required to span the channel. We conclude that LF_N adopts an extended-chain configuration as it translocates; i.e., the channel unfolds the secondary structure of the protein. We also show that the channel not only can translocate LF_N in the normal direction but also can, at least partially, translocate LF_N in the opposite direction.

INTRODUCTION

Anthrax toxin is composed of three proteins: edema factor (EF; 89 kD), lethal factor (LF; 90 kD), and protective antigen (PA; 83 kD). The former two are enzymes that, to carry out their functions, must gain access to the cytosol of targeted cells. This they achieve through the agency of the third component, PA. The sequence of events by which this occurs is the following (for a general review of anthrax toxin, see Young and Collier, 2007). (a) PA binds to a receptor on the cell surface, where it is cleaved by a protease into a 20-kD (PA₂₀) and a 63-kD (PA₆₃) fragment. (b) The latter, which remains bound to the cell surface, oligomerizes to form ring-shaped heptamers (Lacy et al., 2004) and octamers (Kintzer et al., 2009), collectively termed the prepores, and in so doing creates binding sites for EF and LF.

(c) With up to three (for the heptamer) (Melnik et al., 2006) or four (for the octamer) (Feld et al., 2010) of these sites occupied by EF and/or LF, the complexes undergo receptor-mediated endocytosis and ultimately arrive in an acidic vesicle compartment. (d) There the prepore undergoes a conformational change, resulting in its forming a pore (which we shall refer to as a channel) in the endosomal membrane that serves as the conduit for the translocation of EF and LF to the cytosol.

The (PA₆₃)₇ channel is a mushroom-shaped structure with a cap, containing the binding sites for EF and LF and a long 100-Å stem (Katayama et al., 2008) (Fig. 1). When reconstituted into a planar phospholipid bilayer, the (PA₆₃)₇ channel has a conductance of ~55 pS (in 100 mM KCl, pH 5.5), is highly (although not ideally) permeable to univalent cations, and under appropriate voltage and pH conditions translocates EF and LF across the membrane (Krantz et al., 2006). The diameter of the 100 Å-long stem is ~15 Å (Nguyen, 2004), whereas the diameters of EF and LF, even in the molten globular state they assume at pH 5.5 (Krantz et al., 2004), are

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Abbreviations used in this paper: EF, edema factor; HPDP-biotin, N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; LF, lethal factor; MTS-biotin, N-biotinylaminoethyl methanethiosulfonate; PA, protective antigen; TCEP, tris-(2-carboxyethyl)phosphine; YFP, yellow fluorescent protein.

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much larger. Thus, in order for these proteins to pass through the stem, they must unfold, but it is not known to what extent this occurs. Certainly, tertiary structure must be lost, but given that the stem is wide enough to accommodate an α helix with its side chains, it is not clear to what extent secondary structure is preserved. In this paper, we address this issue and report data suggesting that the polypeptide chain can adopt a fully extended conformation as it translocates through the channel's stem.

Rationale and design of experiments

General strategy. With the transmembrane voltage held at +20 mV (cis voltage relative to that of the trans), LF_N (the 263-residue N-terminal end of LF) added to the cis solution enters the $(PA_{63})_7$ channel N-terminal end first and blocks it (Zhang et al., 2004a). If the voltage is then stepped to a larger cis-positive value ($\geq +40$ mV), LF_N is translocated across the membrane through the channel to the trans solution, whereas if the voltage is stepped to a cis-negative value, LF_N exits the channel back to the cis solution, thereby unblocking it (Zhang et al., 2004b). Our general strategy to determine the number of LF_N residues required to span the length of the channel was as follows. We attached a biotin at position 1 and a molecular stopper, which prevents further translocation of LF_N , at position x . After blocking the channel with this construct at +20 mV, and confirming that after the voltage was stepped to +50 or 55 mV the channel was unblocked when the voltage was stepped back to a negative value, we would then add streptavidin to the trans solution. If the distance from 1 to x was long enough to traverse the channel, streptavidin would tightly bind the biotin at position 1, and consequently unblocking of the channel at negative voltages would be prevented. (An idealized current record before and after streptavidin addition is shown in Fig. 2 A.) If, on the other hand, the distance from 1 to x was not long enough to traverse

the channel, unblocking of the channel at negative voltages would be unaffected by the trans streptavidin. By varying the position of x , we would thereby find a cutoff position, p , where for $x > p$, residue 1 can reach the trans side, and for $x < p$, it cannot. To implement this strategy, we used two different types of stoppers, which we now describe.

Yellow fluorescent protein (YFP). The 27-kD Venus YFP was appended as a stopper to the C terminus of truncated LF_N constructs of various lengths, and a biotin was attached at the N terminus (Fig. 2 B). Experiments were then performed as described above in General strategy, and a cutoff position p was determined (see Results), as described there. There are two problems with this approach that make the meaning of the so-determined cutoff position ambiguous. First, because the size of the vestibule leading up to the stem of the $(PA_{63})_7$ channel (Fig. 1) is not known precisely, it is not clear where this stopper "stops" and hence what length is actually being measured. In the two extremes, YFP either could be stopped from entering the vestibule at all, or it could be stopped only when it reaches the beginning of the stem. Second, even if we knew where the YFP was stopped, it turns out that the forces we are applying with our positive voltage pulses could partially unfold it in the region where it is attached to the C terminus of LF_N (see Discussion); hence, an unknown number of its residues could enter the stem. Thus, the actual length of peptide chain in the stem would be longer than that indicated by the cutoff position p . Because of these uncertainties, we resorted to a different stopper described below.

Biotin at positions other than the N terminus. Even though LF_N with a biotin attached to its N terminus is readily translocated through the $(PA_{63})_7$ channel, a biotin attached at most other positions in LF_N severely retards its translocation; with two successive biotins attached, LF_N

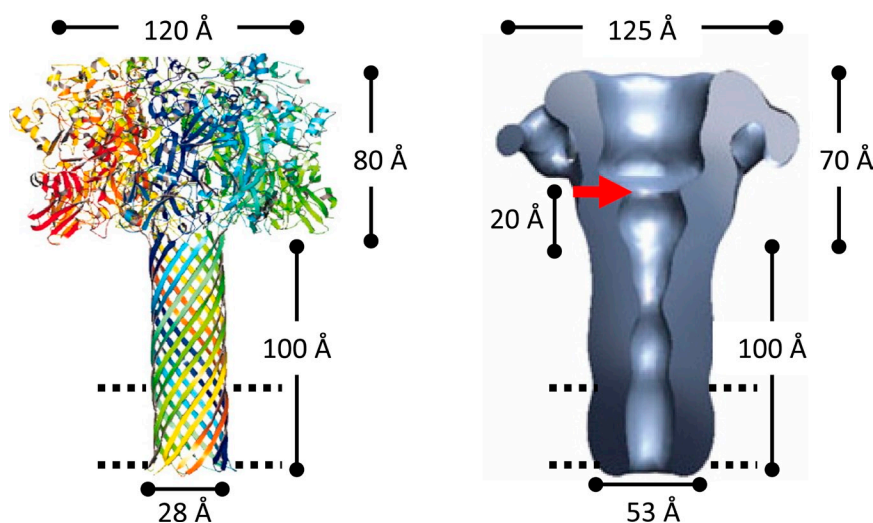


Figure 1. Structure of the $(PA_{63})_7$ channel. Model (Nguyen, 2004) of the $(PA_{63})_7$ channel (left) and its structure (Katayama et al., 2008) as obtained from negative-stain electron microscopy at 25-Å resolution (right). The parallel dashed lines indicate the thickness of the hydrophobic interior of the phospholipid bilayer. The red arrow marks the likely position of the Φ -clamp.

translocation is essentially completely prevented (see Results). Moreover, we have good evidence that the position at which biotin is stopped is at the so-called Φ -clamp (Krantz et al., 2005) that lies near the entrance to the stem (see Results). Constructs were made of whole LF_N , with biotins attached either to residue x or to residues x and $x + 1$, and also with a biotin at its N terminus (Fig. 2 B). The cutoff position p was then determined by experiments as described above in General strategy (see Results).

MATERIALS AND METHODS

Molecular biology and protein purification

A protein with Venus YFP fused, in frame, at the C terminus of LF_N was made as follows. pDBS15 was created by mutating the stop codon of LF_N to a serine codon in the plasmid pET15b- LF_N (Lacy et al., 2002), using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies), and was then digested with BamHI.

A plasmid containing a version of Venus YFP was provided by D. Reeves (ARMGO Pharma, Tarrytown, NY). The gene for Venus YFP with BamHI ends was amplified by PCR, digested with BamHI, and ligated to the BamHI-digested pDBS15 to create pDBS18. pDBS18 thus encodes a 603-residue protein (LF_N -YFP) with LF_N residues 1–263, followed by glycine-serine-glycine, followed by Venus YFP, all with the pET15b-encoded thrombin-cleavable His₆-tag sequence (Merck) at its N terminus.

LF_N 1C-YFP was created by site-directed mutagenesis reactions, using the QuikChange Site-Directed Mutagenesis kit on pDBS18. To delete central portions of LF_N 1C-YFP, complementary mutagenic oligonucleotides were used, with sequences upstream and downstream of those encoding residues subject to deletion, and including the linker residues. Those oligonucleotides were used to delete residues from pDBS18, using the QuikChange Site-Directed Mutagenesis kit, resulting in the various LF_N products: 1–23, 1–33, 1–43, 1–83, and 1–133, all with the YFP attached at the C terminus and the His₆ tag attached at the N terminus. All the LF_N cysteine mutants were created by the QuikChange Site-Directed Mutagenesis kit and contained the same His₆ tag at the N terminus.

Wild-type PA (83 kD), PA R178A/K214E, LF_N , and LF_N -YFP (residues 1–263 of LF, containing the N-terminal His₆ tag) were

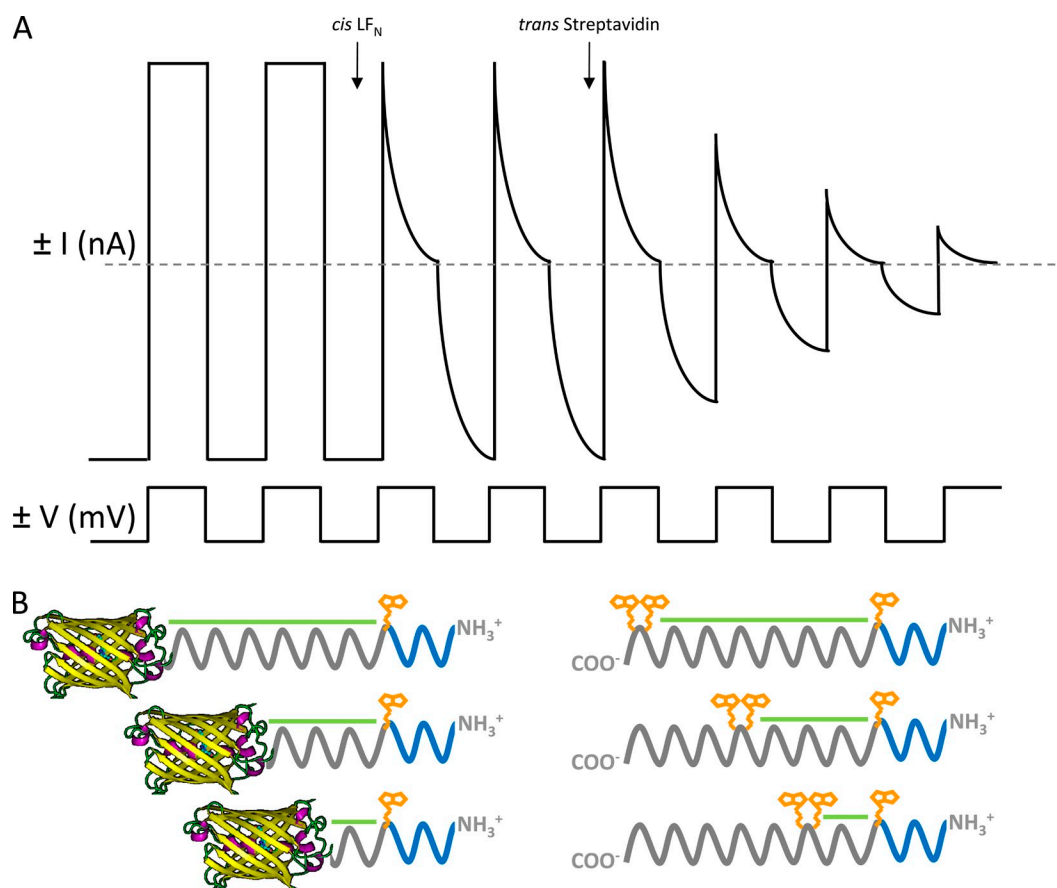


Figure 2. Blocking of $(PA_{63})_7$ channels by LF_N (with a biotin at position 1 and a molecular stopper at position x) at positive voltages and unblocking at negative voltages. (A) Idealized current record. There is no gating of the channels before LF_N addition to the cis solution. After addition, the channels are blocked by positive voltage pulses and become completely unblocked at negative voltage pulses. After the subsequent addition to the trans solution of streptavidin, which was able to bind the biotin at position 1, unblocking at negative voltages is no longer complete and diminishes over time. The dashed line is the zero current level. (B) Cartoons of LF_N constructs with a biotin at position 1 and either a YFP stopper (left) or a double-biotin stopper (right). LF_N is depicted as a wavy gray line, biotins are in orange, the N-terminal (His)₆ tag is in blue, and YFP is depicted as a yellow β barrel. The green line indicates the distance from the stopper to the biotin at position 1. Note that the double-biotin stopper is attached at various positions on full-length LF_N . In contrast, the YFP stopper is at the C-terminal end of various LF_N s, whose C-terminal ends have been truncated.

expressed recombinantly and purified as described previously (Krantz et al., 2004, 2005; Pimental et al., 2004; Zhang et al., 2004b). We inferred from the fact that the purified LF_N-YFP is bright yellow that the YFP moiety is correctly folded in these constructs. The heptameric prepore form of PA₆₃ was prepared by nicking PA₈₃ with trypsin and purifying the PA₆₃ heptamer from the smaller 20-kD fragment using anion-exchange chromatography (Cunningham et al., 2002).

Biotinylation of (His)₆-LF_N mutants

The LF_N cysteine mutants were reduced with 20 mM dithiothreitol and then dialyzed into degassed 150 mM NaCl and 20 mM Tris, pH 8.5. 100 μ l of a given LF_N mutant (2 mg/ml) was incubated with 3 μ l N-biotinylaminoethyl methanethiosulfonate (MTS-biotin; 20 μ g/ μ l; Toronto Research Chemicals) for 30 min at room temperature. (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide [HPDP-biotin] labeling, which was done only for the experiment shown in Fig. S1 B, was similar to that of MTS-biotin, except that the incubation was done for 3 h at 30°C; Qiu et al., 1996.) The reaction was stopped by dialyzing out the biotinylating reagent at 4°C. To confirm that virtually all of the protein was labeled, biotin-reacted and unreacted protein were incubated with streptavidin. The biotin-reacted LF_N exhibited a shift on the gel, whereas the unreacted protein was unaffected (Fig. 3); wild-type LF_N, which has no native cysteines, was also unaffected (not depicted).

Planar lipid bilayers

Bilayers were formed by the brush technique (Mueller et al., 1963) across a 250- μ m diameter aperture in a polystyrene cup (Wonderlin et al., 1990). Membranes separated two compartments

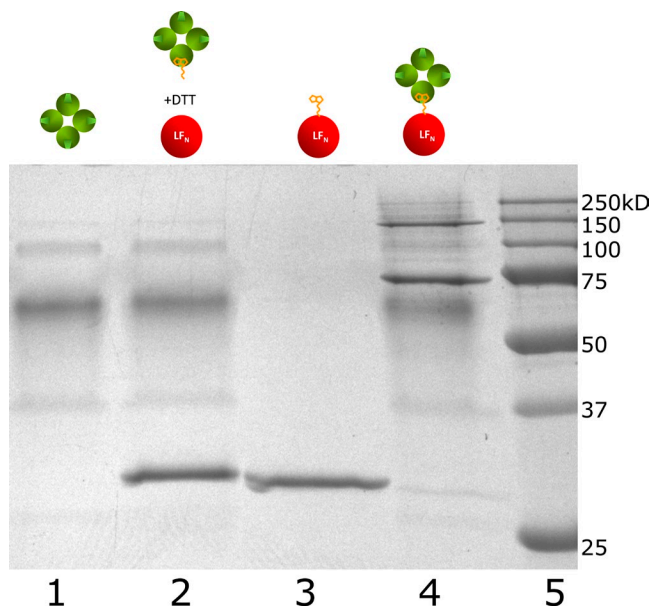


Figure 3. Example of an SDS nonreducing 15% polyacrylamide gel of an LF_N mutant with cysteines at residues 1 and 126. Lane 1, streptavidin; lane 2, the LF_N mutant reacted with MTS-biotin, incubated with streptavidin, and then subsequently treated with dithiothreitol (DTT); lane 3, the LF_N mutant reacted with MTS-biotin; lane 4, the LF_N mutant reacted with MTS-biotin and then incubated with streptavidin. Note the gel shift in lane 4 caused by the incubation with streptavidin. In the cartoons above each lane, the four green balls represent tetrameric streptavidin and the orange rings and tail represent biotin; only one of the two biotins (one at residue 1 and one at residue 126) that were introduced by the reaction of LF_N mutant with MTS-biotin is depicted.

of 1 ml containing symmetric buffered solutions of 100 mM KCl, 5 mM potassium succinate, and 1 mM EDTA, pH 5.5, which could be stirred by small magnetic bars. Agar salt bridges (3 M KCl, 3% agar) linked Ag/AgCl electrodes in saturated KCl baths to the cis and trans compartments. The membrane-forming solution was 3% diphytanoyl-phosphatidylcholine (Avanti Polar Lipids, Inc.) in *n*-decane, and membrane formation was monitored both visually and by capacitance (\sim 500 pF). All experiments were done under voltage clamp conditions with a Bilayer Clamp (BC-535C; Warner Instruments); voltages are those of the cis solution (to which protein was added) with respect to the trans solution, which was held at virtual ground. Current responses were filtered at 3 kHz by a low-pass eight-pole Bessel filter (Warner Instruments), recorded by computer via an analogue-to-digital converter (NI USB-6211; National Instruments) at 20 Hz using IGOR NIDAQ Tools MX 1.0 and IGOR 6.0.3.1 (WaveMetrics), and recorded by a DMP-4B Physiograph chart recorder (Narco Bio-Systems, Inc.) for annotation purposes.

(PA₆₃)₇ channel formation and (His)₆-LF_N conductance block

After membrane formation, PA₆₃ prepore heptamer was added to the cis compartment (to a final concentration of \sim 1 μ g/ml [\sim 2 nM]), which was held at a $\Delta\psi$ of +20 mV with respect to the trans compartment. When the conductance resulting from (PA₆₃)₇ channel formation reached an appropriate level (generally after \sim 30 s), the cis compartment was perfused, which removed the (PA₆₃)₇ from solution and caused the conductance to level off. Perfusion was accomplished using a BPS-2 Bilayer Perfusion System (Warner Instruments) coupled to a pair of syringes; 10 vol were exchanged in 3 min. LF_N or one of its constructs was then added to the cis compartment (final concentration of \sim 120 nM). The progress of LF_N binding to (PA₆₃)₇ channels and blocking them was monitored by the continuous fall of conductance.

Translocation experiments

After LF_N conductance block of (PA₆₃)₇ channels was complete, excess LF_N was removed from the cis compartment by perfusion, while $\Delta\psi$ was held constant at +20 mV. After this, $\Delta\psi$ was stepped to a larger voltage (e.g., +50 mV), and the rate of LF_N translocation was determined, as reflected in the rate of conductance rise caused by LF_N traversing the channel and exiting into the trans solution. In general, the final conductance was \geq 90% of the conductance before blocking by LF_N.

For the N-terminal biotin-reacted LF_N 1–263 constructs containing a stopper, after the normally translocating positive voltage was applied for \sim 60 s, and little or no translocation was observed, we repeatedly stepped the voltage for \sim 60 s to a negative voltage, and then back to +20 mV, to confirm that these channels were blocked and had not simply disappeared. Negative voltages drive LF_N out of the channel back to the cis side, so that the conductance immediately obtained at +20 mV (before the slow reblocking of the channels by still-attached LF_N) represents that of unblocked channels. The conductance ultimately obtained was \geq 90% of the conductance level before blocking by LF_N.

Streptavidin binding experiments

After LF_N block of (PA₆₃)₇ channels was complete at +20 mV, the pulsing protocol began with a negative voltage step, in which we monitored the unblocking of the LF_N, followed by a positive voltage step, in which the LF_N reblocking was observed. This was repeated several times to obtain a stable “baseline” value of unblocking. After this level was attained, we proceeded to add streptavidin to the trans compartment to a final concentration of 50 μ g/ml (1 μ M) while maintaining the pulsing protocol. We then observed whether or not the baseline of unblocking was reduced after the streptavidin addition, indicating that the N-terminal biotin in LF_N had or had not been grabbed.

Online supplemental material

Fig. S1 describes two control experiments that confirm that the trans action of streptavidin is indeed the binding of the N-terminal biotin in the LF_N construct 1–83 YFP: (1) stopping the reaction with an excess of free biotin, and (2) reversing the reaction with the addition of TCEP that reduces the S–S bond between the cysteine at position 1 in LF_N and the HPDP-biotin. Fig. S1 is available at <http://www.jgp.org/cgi/content/full/jgp.201010578/DC1>.

RESULTS

Intrinsic gating of $(PA_{63})_7$ channels

Before presenting our data on the trapping of LF_N constructs within the $(PA_{63})_7$ channel, we first consider the voltage-gating behavior of these channels. By doing so, we hope the reader will more clearly understand the records shown in the following sections. As has been reported, $(PA_{63})_7$ channels tend to “turn off” at both positive and negative voltages, with the degree and rapidity of turn off much greater at negative voltages (Blaustein et al., 1989; Anderson and Blaustein, 2008). We see illustrations of this in Fig. 4. The mechanism of this voltage gating is not understood, although an interesting study has recently been made of the gating at negative voltages (Anderson and Blaustein, 2008). Moreover, the magnitude of the gating varies unpredictably from membrane to membrane; thus, in some membranes modest slow turn off occurs at -60 mV, whereas in others rapid and almost complete turn off occurs at -20 mV. This turn off complicates the kinetics of unblocking at

negative voltages of LF_N -treated membranes. Something approaching the idealized record in Fig. 2 A is achieved when the $(PA_{63})_7$ gating is slow (Fig. 4 A). In most cases, the full unblocking of LF_N at negative voltages is obscured by the concurrent intrinsic closing of the channels at those voltages (Fig. 4 B). We routinely checked the gating behavior of a $(PA_{63})_7$ -treated membrane before adding LF_N , and on that basis we chose the lengths of times we held the voltage at positive and negative potentials. This was strictly an aesthetic decision; it did not affect the main conclusion drawn from an experiment, namely, whether trans streptavidin did or did not grab the N-terminal biotin in LF_N .

YFP as the stopper

After establishing, as expected, that YFP attached to the C terminus of LF_N prevented translocation (Fig. 5), we proceeded to test truncated LF_N constructs to determine if the N terminus reached the trans solution. A representative example is shown in Fig. 6. It is clear that trans streptavidin grabbed the N-terminal biotin of LF_N that had been truncated to 83 residues. The results of these experiments are summarized in Fig. 7, where we see that 23 residues are insufficient to traverse the channel, whereas 33 residues or more are sufficient. The cut-off appears to be close to 33 residues (see Fig. 7).

A confirmation that the reason that trans streptavidin prevented unblocking of the channels at negative voltages, as seen in Fig. 6, was because it bound the N-terminal biotin would be to show that an excess of free biotin

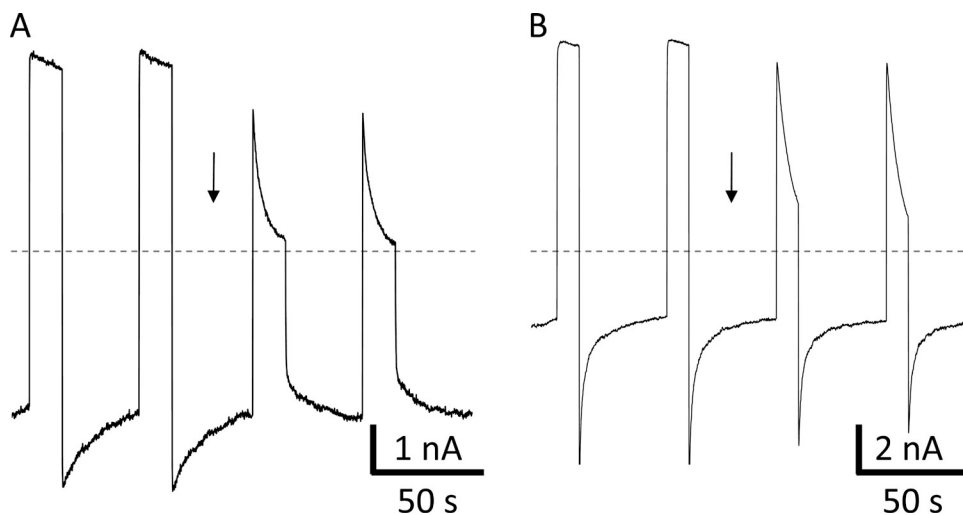


Figure 4. Records illustrating the voltage gating of $(PA_{63})_7$ channels and its effect on the LF_N unblocking kinetics. Before $(His)_6$ - LF_N addition to the cis solution, there is some gating of the channels at positive voltage pulses and very significant gating at negative voltage pulses; the latter is more marked in B than in A. The gating kinetics are such that channels that have closed at negative voltages rapidly reopen at positive voltages. After LF_N addition (arrow), blocking occurs at positive voltage pulses, but the unblocking kinetics at negative voltage pulses are convoluted with the intrinsic gating kinetics of the channels. In A, actual un-

blocking at negative voltage can be seen. In B, unblocking at negative voltage pulses is totally obscured by the intrinsic gating kinetics of the channels. Nevertheless, the fact that unblocking has occurred at negative voltages is revealed when the voltages are stepped back to positive values. Note that in both A and B, complete unblocking has not occurred during the negative voltage pulses, as the initial current when the voltage is stepped back to positive values is smaller than the current before LF_N addition. The dashed line in A and B marks the zero current level. Before the start of each record, $(PA_{63})_7$ was added to the cis compartment to a concentration of 2 nM, with the voltage held at $+20$ mV, and after an appropriate level of current was reached, the cis compartment was perfused, which removed free $(PA_{63})_7$ from the solution and caused the current to level off. The voltage protocol of $+45$ and -50 mV was then begun. At the arrows, the $(His)_6$ - LF_N construct 1–83, with YFP attached to the C terminus and biotin attached at residue 1, was added to a concentration of 120 nM.

stopped the reaction and/or that unblocking was restored when the biotin, with its attached streptavidin, was removed from the N terminus by the disulfide-reducing agent tris-(2-carboxyethyl)phosphine (TCEP). We were successful in demonstrating the former (Fig. S1A), but we were initially unsuccessful in demonstrating the latter. The reason for this, we felt, was that the disulfide linker of MTS that attaches the biotin to the N terminus of LF_N is so short that the disulfide bond is buried in the streptavidin-binding pocket and is thereby made inaccessible to TCEP (Fig. S1 B, inset). That this was indeed the explanation was shown when we attached the biotin with the longer linker HPDP-biotin. In this case, unblocking was rapidly restored by the trans addition of TCEP (Fig. S1 B).

The kinetics of trans streptavidin engaging the N-terminal biotin are a function of both the magnitude of the voltage pulse driving the LF_N chain through the $(PA_{63})_7$ channel and the length of that pulse. The larger the magnitude of the pulse and the longer its duration, the faster the kinetics. Taking again the 83-residue truncated construct as representative, we see that for a

given pulse length (5 s), the rate of streptavidin grabbing increases about e-fold per 5.5 mV (Fig. 8 A), and that for a given voltage (+40 mV), the rate increases about e-fold per 8-s pulse duration (Fig. 8 B). It is intuitively obvious why the rate of streptavidin binding to the N-terminal biotin should increase with voltage and pulse duration, as both of these factors contribute to the length of time that the N-terminal biotin was exposed to the trans solution. Why the rate should increase with the length of the LF_N construct (Fig. 7), although not so obvious, has a ready explanation. Namely, as a result of Brownian motion of the peptide chain, the N terminus must fluctuate between the times it is exposed to the trans solution and the times it has withdrawn into the channel. The longer the length of the constructs, the longer the fraction of time that the N-terminal residue spends exposed to the trans solution.

Biotin as the stopper

The introduction of a biotin at most positions in LF_N (the N terminus excepted), or at two successive positions, virtually completely stopped LF_N translocation through the

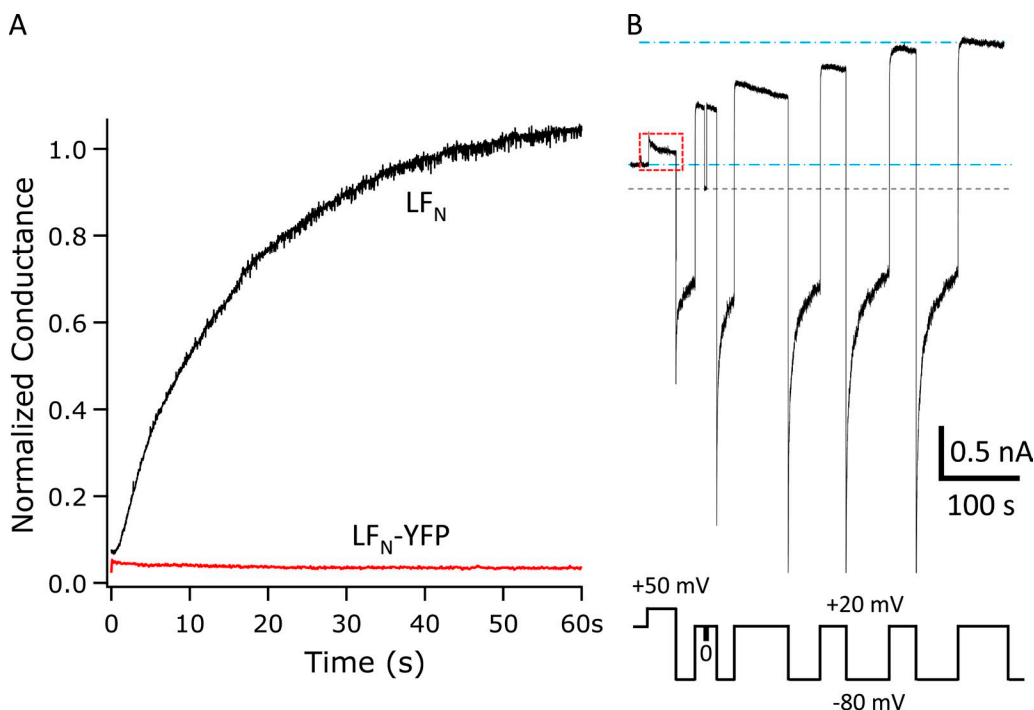


Figure 5. Demonstration that YFP attached to the C terminus of LF_N prevents its translocation. (A) Before the start of the records, the $(PA_{63})_7$ -induced conductance had leveled off, $(His)_6$ - LF_N or $(His)_6$ - LF_N -YFP had been added to the cis compartment to a concentration of 120 nM, the conductance had fallen to a fraction of the value before LF_N addition, and LF_N had been perfused out of the cis compartment. At $t = 0$, the voltage was stepped from +20 to +50 mV. We see that LF_N was readily translocated, with a half-time of ~ 10 s, whereas no translocation at all of LF_N -YFP had occurred after more than 60 s. The conductance is normalized to the value before the addition of LF_N or LF_N -YFP. (B) The LF_N -YFP record in A is a blow-up of the boxed segment of this record. The point of the rest of the record is to show that the $(PA_{63})_7$ channels are still present and have not simply disappeared. The -80 -mV pulses drive LF_N out of the channel back to the cis side. The initial conductance when the voltage is stepped back to +20 mV is a result of the channels that were unblocked at -80 mV; the subsequent slow fall in conductance is caused by LF_N still attached to those channels. The dashed blue lines mark the conductance before and after LF_N -YFP block. We see that after five -80 -mV pulses, the conductance has reached the value before LF_N -YFP addition, thus confirming that $(PA_{63})_7$ channels have not disappeared. The black dashed line is the zero current level.

(PA₆₃)₇ channel (Fig. 9). A priori we suspected that the biotins were stopped at the Φ -clamp (which is near the entrance to the stem of the channel) for the following reasons. When the phenylalanines of the Φ -clamp were mutated to alanines, the single-channel conductance doubled (Krantz et al., 2005), suggesting that a restriction in channel diameter occurs at the Φ -clamp that could impede the passage of biotin past it. In addition, the Φ -clamp was identified as the binding site for hydrophobic groups (Krantz et al., 2005), which suggests that it could hinder biotin movement by binding it. That the biotins were indeed stopped at the Φ -clamp was confirmed by the observation that when the phenylalanines of the Φ -clamp were mutated to alanines, LF_Ns with introduced biotins were readily translocated (Fig. 9). With this established, we proceeded to test whole LF_Ns with biotins introduced at various positions to determine if the N-terminal biotin reached the trans solution. We con-

firmed in a separate experiment for each biotinylated LF_N that it was not translocated (e.g., as in Fig. 9).

Before presenting the results of these experiments, we wish to make a point concerning a difference in methodology between these experiments with biotin as the stopper and those previously described with YFP as the stopper. With the exception of LF_N 1–263, all the experiments with the YFP stopper, as depicted in Fig. 7, were done on wild-type (PA₆₃)₇ channels, whereas all the experiments to be described with the biotin stopper were done on the double-mutant (PA₆₃)₇ channel R178A/K214E. Why was this? Because none of our truncated LF_N mutants contained the region that binds to (PA₆₃)₇ in the mushroom cap (Melnik et al., 2006), unblocking at negative voltages was fairly rapid. In contrast, unblocking at negative voltages of whole LF_N, which contains the region that binds to (PA₆₃)₇, was extremely slow (unpublished data). (This is probably mainly a

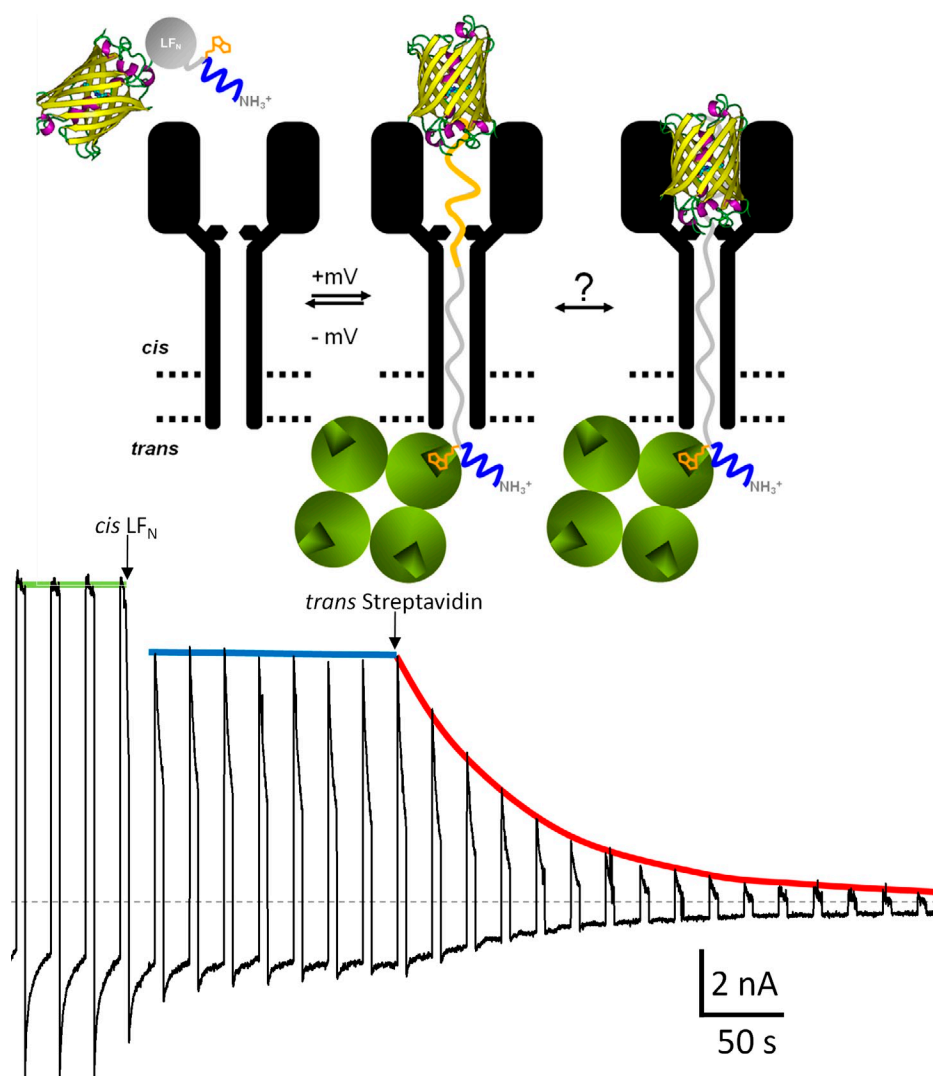


Figure 6. Example of a truncated (His)₆-LF_N construct (LF_N 1–83), whose N terminus reached the trans solution. Before the start of the record, (PA₆₃)₇ was added to the cis compartment to a concentration of 2 nM, with the voltage held at +20 mV; after an appropriate level of current was reached, the cis compartment was perfused, which removed the (PA₆₃)₇ from the solution and caused the current to level off. The voltage protocol of +80 mV for 5 s and –40 mV for 15 s was then instituted. At the first arrow, the LF_N construct 1–83, with YFP attached to the C terminus and biotin attached at residue 1, was added to the cis solution to a concentration of 120 nM, and a constant level of unblocking was obtained. At the second arrow, streptavidin was added to the trans compartment to a concentration of 1 μM. We see a dramatic decrease of unblocking over time, thus demonstrating that the biotin at residue 1 had reached the trans solution and had been grabbed by streptavidin, thereby preventing those channels from becoming unblocked at –40 mV. The dashed line is the zero current level. In the cartoon, YFP is depicted as a β barrel (Rekas et al., 2002), biotin is in orange, the N-terminal (His)₆ tag is in blue, streptavidin is represented by four green balls, and the (PA₆₃)₇ channel is depicted in black with a wide opening to the cis solution and the entrance to the long stem of the channel marked by the Φ -clamp.

The ambiguity of where YFP is stopped is depicted by two extremes: in the first, it cannot enter the channel at all, but its N-terminal end may partially unfold as depicted by the wavy yellow line; in the second, it is stopped at the Φ -clamp. The parallel dashed lines indicate the thickness of the hydrophobic interior of the phospholipid bilayer.

consequence of the fact that in addition to the LF_N that is blocking the channel, there are one or two other LF_Ns bound to the cap, and these may sterically and/or electrostatically interfere with the exiting of the blocking LF_N from the channel at negative voltages.) To circumvent this problem, we used the above-mentioned double-mutant (PA₆₃)₇ channel that does not bind LF_N in the cap (Melnik et al., 2006; Feld et al., 2010).

Two examples of the experiments with successive biotins as the stopper are shown in Fig. 10. We see that trans streptavidin grabbed the N-terminal biotin with the biotin stoppers at positions 34 and 35, whereas it did not grab the N-terminal biotin with the biotin stoppers at positions 32 and 33. The results of these experiments are summarized in the Fig. 10 inset, where we see that 31 residues are insufficient to traverse the stem of the channel, whereas 33 residues or more are sufficient. There is a sharp cutoff between 31 and 33 residues.

Reverse translocation

The experiments we have described of adding LF_N constructs to the cis solution and streptavidin to the trans

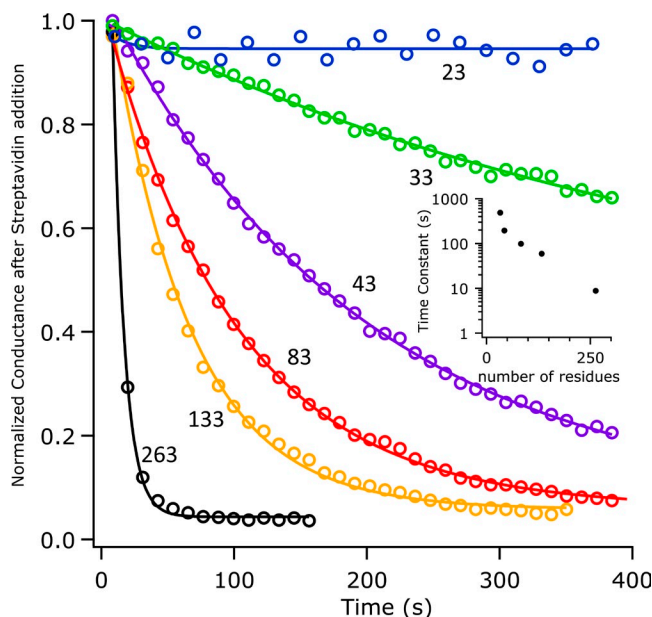


Figure 7. The rate of capture by trans streptavidin of the biotin at residue 1 of (His)₆-LF_N constructs of various lengths, with YFP attached to the C terminus. These data come from experiments such as that depicted in Fig. 6 and are fit by single exponentials with the time constants indicated in the inset. The conductances are normalized to the value before the addition of LF_N-YFP. The voltage protocol for all of these records was +50 mV for 5 s and −40 mV for 15 s. Note that, as expected (see Results and Discussion), the shorter the length of the construct, the longer the time constant. Most significantly, the biotin at residue 1 of LF_N 1–23 is not captured by trans streptavidin, whereas that for LF_N 1–33 is slowly captured. (The LF_N 1–263 experiment was done on channels formed by the PA₆₃ double mutant R178A/K214E.) The time on the abscissa is the cumulative time spent at positive and negative voltages.

were also performed in the opposite direction, i.e., adding LF_N constructs to the trans solution and streptavidin to the cis. In this latter case, the voltage protocol was reversed, and the channels were blocked by negative voltages and unblocked by positive voltages. Whereas with LF_N added to the cis solution the unblocking kinetics at negative voltages are convoluted with the intrinsic gating kinetics of (PA₆₃)₇ channels, with LF_N added to the trans solution the blocking kinetics at negative voltages are convoluted with the intrinsic gating kinetics of the channels. Nevertheless, it is clear that cis streptavidin can grab the N-terminal biotin (Figs. 11 and 12), thereby demonstrating that the channel can translocate LF_N in either direction. Incidentally, this is the first demonstration that the (PA₆₃)₇ channel can, at least partially, translocate LF_N in the backward direction, but still, as in the forward direction, N-terminal end first. We tested the same LF_N constructs in these trans-addition experiments as in the cis-addition experiments. Using biotins as the stopper, we could say that 28 residues are sufficient to reach the cis solution from the Φ-clamp, but 20 residues are not (Fig. 11, inset). (We did not attempt to narrow the cutoff as finely in these LF_N experiments as we did in the cis LF_N experiments.) However, because 20 residues have an extended length of 72 Å, but the distance from the Φ-clamp to the cis solutions is ~50 Å (Fig. 1), this suggests that the biotin stopper is stopped somewhat short of the Φ-clamp. Using YFP as the stopper, we see that 43 residues are sufficient to traverse the entire channel length from the trans side to the cis side, but 33 residues are not (Fig. 12).

DISCUSSION

Given the structure of the (PA₆₃)₇ channel, with its ~15 Å-wide, 100 Å-long stem (Fig. 1), it is obvious that for a globular protein to be translocated through it, the protein has to unfold. Certainly, tertiary structure must be lost, but it is not clear to what extent secondary structure is preserved, given that the stem is wide enough to accommodate an α helix with its side chains. In this paper, we have addressed this question for LF_N, the 263-residue N-terminal fragment of LF, by determining how many of its residues are required to span the length of the channel. We did this by attaching a biotin at LF_N's N terminus, which is the end that first enters the channel on its way to being translocated, and a molecular stopper elsewhere in the molecule. After adding streptavidin to the opposite side of the membrane (the trans side) from that to which LF_N was added (the cis side), we then applied a voltage (~50 mV) that would normally cause LF_N to be translocated (were it not for the stopper). We established by its binding or not binding by streptavidin whether the N-terminal biotin had reached the trans solution. By varying the distance between the stopper and the N-terminal biotin, we thereby determined

the minimum number of residues required to span the length of the channel. Note that the linker attaching biotin to the N terminus of LF_N is very short because the biotin was attached through the reaction of the cysteine with MTS-biotin (see Fig. S1 B, inset). Thus, the cysteine (introduced by site-direct mutagenesis) at the N terminus of LF_N must reach the trans solution in order for streptavidin to bind to its attached biotin.

The number of residues spanning the channel

We used two different stoppers and LF_N constructs to determine this minimum number of residues. One was YFP attached to the C terminus of truncated LF_N constructs having a biotin at the N terminus. The other was whole LF_N with one or two adjacent biotins attached at various distances from the biotin-containing N terminus. Both stoppers gave essentially the same minimum number of residues required to span the length of the channel. Namely, the YFP stopper gave a number slightly fewer than 33 residues (actually fewer than 36 residues including the linker) (Fig. 7), and the biotin stopper gave a number of 32 or 33 residues (Fig. 10). How are we to interpret these numbers, and what does their agreement imply?

Let us first turn to the biotin stopper result, whose interpretation we feel is less ambiguous. It is fairly certain that the biotin stoppers stop at the Φ -clamp (Fig. 9), and thus, 32 or 33 residues are the minimum number of residues required to span the distance from the Φ -clamp

to the trans solution. Because the Φ -clamp is probably near the entrance to the stem of the mushroom-shaped (PA₆₃)₇ channels (Fig. 1), these 32 or 33 residues are spanning a distance of ~ 120 Å (Fig. 1). As an α helix, with a rise of 1.5 Å per residue, these residues would only span a distance of ~ 50 Å. On the other hand, as an extended chain of ~ 3.6 Å per residue (Dickerson and Geis, 1969), the distance spanned by these residues would be 115–119 Å. We therefore can conclude that the first 33 residues of LF_N traverse the β -barrel stem of the channel in a largely extended configuration.

There is an unstated assumption in the above conclusion, and indeed in all of our experiments. Namely, we have tacitly assumed that streptavidin is sampling a representative state of LF_N and not some rare, unusual state. Thus, a possible interpretation of our results might be that the LF_N chain within the channel is predominantly α helical, that it only rarely adopts an extended configuration, and that it is this rare event that streptavidin captures. Although we certainly acknowledge that the extended chain need not be rigidly fixed and that it could relax to a more ordered state (e.g., a part of it occasionally adopting an α -helical structure), we feel that our data are consistent with the chain spending most of the time extended. Consider Fig. 7, for example, and in particular compare the rate of binding of streptavidin to LF_N 1–83 to that to LF_N 1–43. The time constant for the former is ~ 100 s and that for the latter is ~ 200 s (see inset). (Although not directly relevant

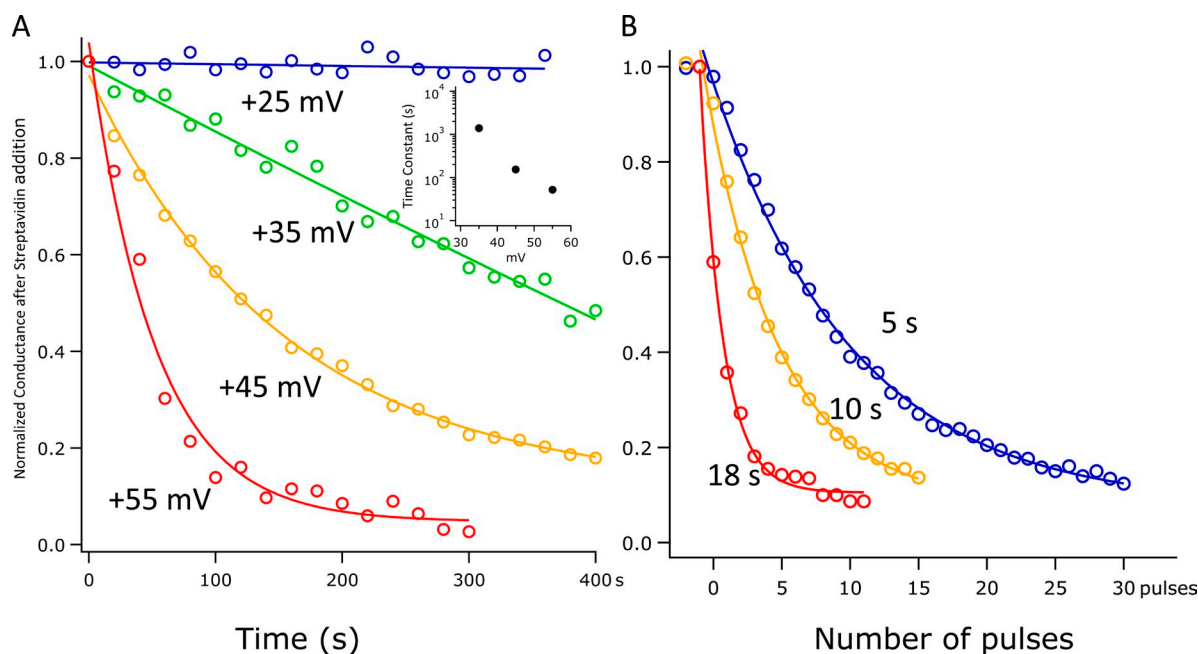


Figure 8. The rate of capture by trans streptavidin of biotin at residue 1 is a function of both the magnitude of the voltage pulse driving the LF_N chain through the (PA₆₃)₇ channel (A) and the length of the pulse (B). The data in all of these records were obtained with the construct LF_N 1–83 from experiments such as that depicted in Fig. 6. In A, the positive voltage pulse duration was 5 s; in B, the magnitude of the voltage pulse was +40 mV. The data points are fit by single exponentials. The conductances are normalized to the value before the addition of LF_N 1–83.

to the argument that follows, the reader may note that because in these experiments the positive translocating voltage was applied for only 25% of the time [see figure legend], the actual time constants are 25 and 50 s, respectively.) Now assume that the LF_N within the channel is an α helix. LF_N 1–83 would be about long enough (123 Å) to span the distance from the Φ -clamp to the trans solution (~ 120 Å) and thus always be exposed to the streptavidin in the trans solution. For LF_N 1–43 with an α -helical length of 63 Å to be grabbed by streptavidin at a rate only twofold slower than that for LF_N 1–83, the entire LF_N chain within the channel would have to spend approximately half of its time almost completely unfolded and half of its time α helical. We think this is most unlikely, and that a much more reasonable explanation for the difference in time constants lies in the difference in the time of exposure to the trans solution of extended chains of 43 and 83 residues undergoing Brownian motion. That is, because of the Brownian motion of an extended chain, its N terminus will be drawn back into the channel where it is not exposed to the trans solution. The longer the chain, the less time its N terminus will be found in this retracted state, and hence the more time it will be exposed to the trans solution.

What do we make of the fact that we obtain the same result with the YFP stopper? The simplest interpretation is that it too stops at the Φ -clamp (see Fig. 6, cartoon). Because YFP is cylindrical in shape with a length of ~ 40 Å and a width of ~ 30 Å (Rekas et al., 2002), this would mean that the vestibule leading to the Φ -clamp is quite wide, at no place narrower than 30 Å. In the absence, at present, of structural data to contradict this, we are free to draw this conclusion. Another, less appealing interpretation is that the agreement of the YFP stopper result with that of the biotin stopper is coincidental; that, in fact, YFP cannot enter the vestibule at all but can partially unfold to extend a part of itself from the vestibule entrance to the Φ -clamp (see Fig. 6, cartoon). Although unlikely, this possibility cannot be dismissed out of hand. The first 33 residues of LF_N contain seven lysines, two arginines, and two histidines, which at pH 5.5 (the pH of our experiments) represent ~ 10 positive charges. Assuming that aspartates and glutamates within these 33 residues are protonated (Krantz et al., 2006; Finkelstein, 2009), this means that there are 10 positive charges in the channel's stem. Assuming that the ~ 50 mV that we applied in our experiments drops linearly down the 100-Å stem, this translates into a force of 8 pN being

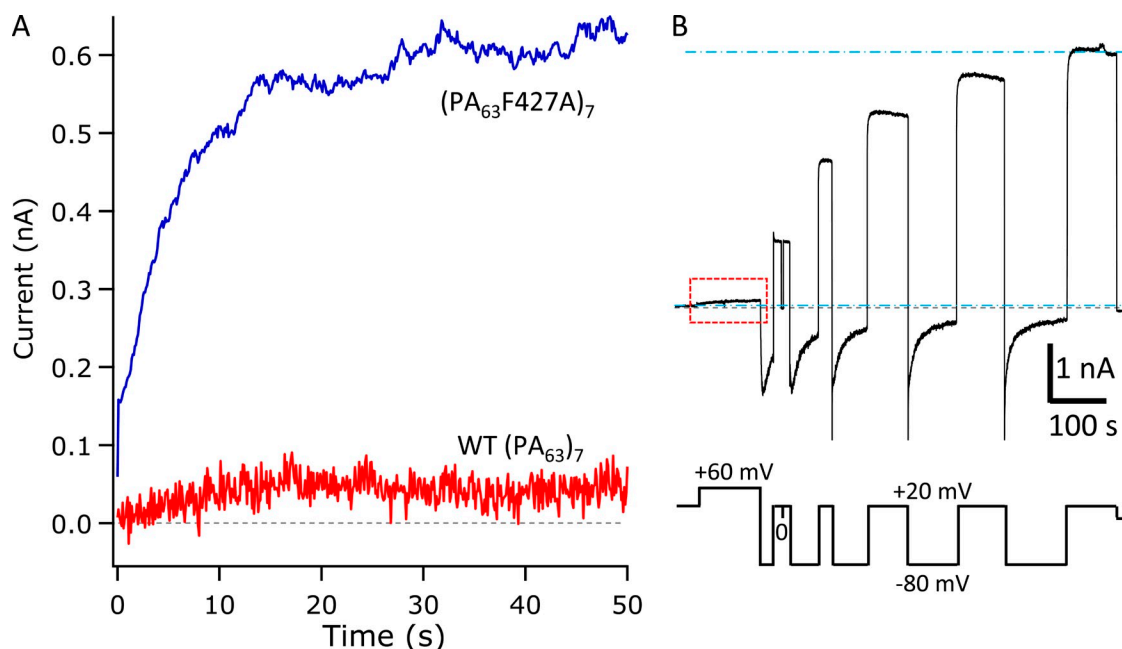


Figure 9. Biotins introduced at most positions in LF_N stop LF_N translocation because the biotins cannot get past the Φ -clamp. The data depicted come from experiments with the $(His)_6$ - LF_N 1C/242C mutant that had been reacted with MTS-biotin to attach biotins at those sites. (A) Before the start of the record, the $(PA_{63})_7$ -induced conductance had leveled off, LF_N had been added to the cis compartment to a concentration of 120 nM, the conductance had fallen to a fraction of the value before LF_N addition, and LF_N had been perfused out of the cis compartment. At $t = 0$, the voltage was stepped from +20 to +60 mV. We see that for the wild-type $(PA_{63})_7$ channel, there was no translocation of LF_N with a biotin attached at residue 242, whereas for the $(PA_{63}F427A)_7$ channel, where the phenylalanines of the Φ -clamp have been mutated to alanines, translocation readily occurred, thus indicating that the biotin at residue 242 could not get past the Φ -clamp. The dashed line is the zero current level. (B) The wild-type $(PA_{63})_7$ channel record in A is a blow-up of the boxed segment of this record. As in Fig. 5 B, the point of the rest of the record is to show that the $(PA_{63})_7$ channels are still present and have not simply disappeared. (See legend to that figure for an exegesis of this one.)

transmitted back to the YFP. This magnitude of force can cause partial unfolding of YFP (Perez-Jimenez et al., 2006). It is by no means clear if the force being transmitted to YFP in our experiments is acting at the same location as in the Perez-Jimenez et al. (2006) experiments. Nor is it clear if the assumption that the applied voltage drops linearly in the stem (as opposed, for example, to the idea that the entire voltage drop occurs across the Φ -clamp) is valid. We are merely pointing out

that we cannot exclude, a priori, the unfolding of YFP as a complicating factor in our experiments.

The results of the reverse translocation experiments, in which we added LF_N to the trans side instead of to the cis side, nicely complement those results. In particular, the experiments with YFP as the stopper are especially revealing. Because YFP is much too large to enter the channel from the trans side, the distance measured in these experiments should give the entire length of the

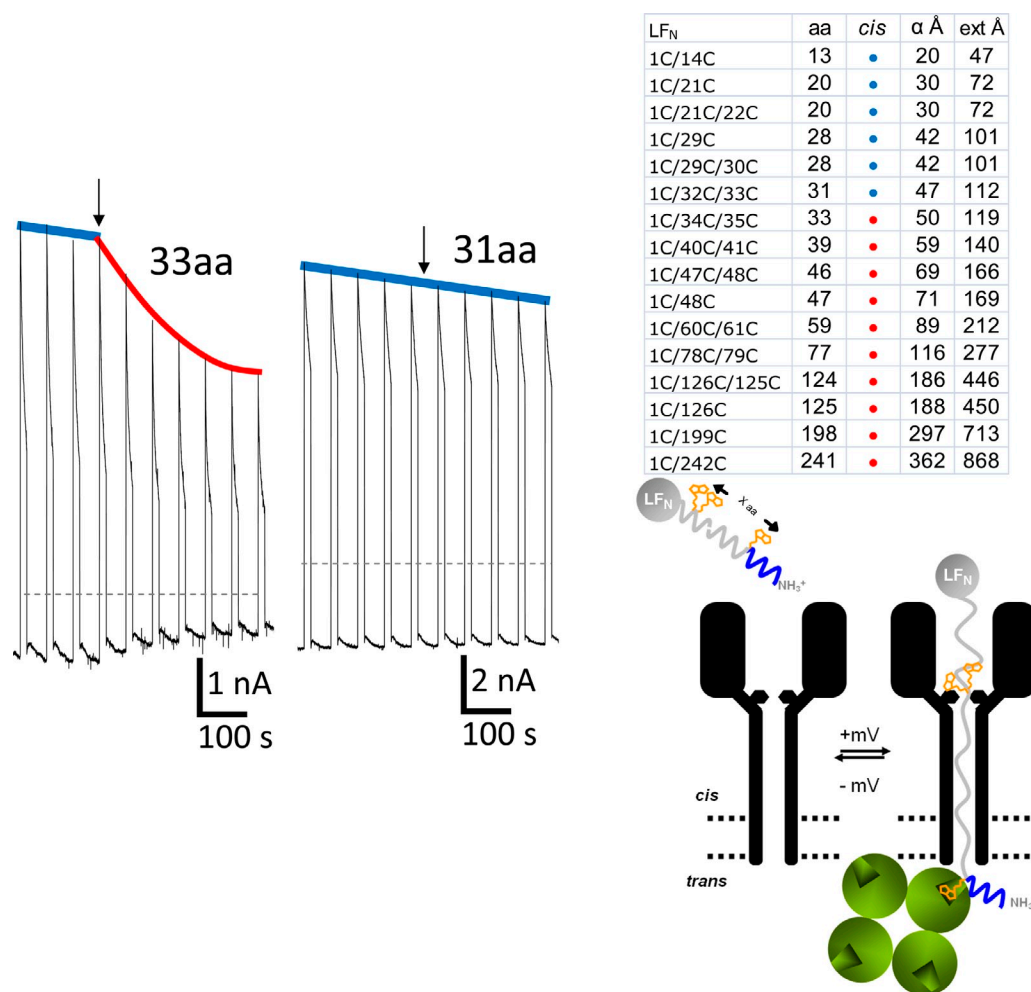


Figure 10. Example of a $(\text{His})_6\text{-LF}_N$ construct (LF_N 1C/34C/35C with these residues biotinylated) whose N terminus reached the trans solution, and a $(\text{His})_6\text{-LF}_N$ construct (LF_N 1C/32C/33C with these residues biotinylated) whose N terminus did not reach the trans solution. Before the start of the records, $(\text{PA}_{63})_7$ with the mutation R178/K214E was added to the cis compartment to a concentration of 2 nM, with the voltage held at +20 mV, and after an appropriate level of current was reached, the cis compartment was perfused, which removed free $(\text{PA}_{63})_7$ from solution and caused the current to level off. The relevant LF_N cysteine mutant was then added to the cis solution to a concentration of 120 nM, and after the fall in conductance had leveled off, the voltage protocol of +55 mV for 12.5 s and -20 mV for 37.5 s was begun. At the arrows, streptavidin was added to the trans compartment to a concentration of 1 μM . In both records, we see that before the addition of streptavidin, the level of unblocking was slowly decaying, having not yet reached a steady state. Most significantly, however, is that in the case of LF_N 1C/34C/35C, this rate of decline increased dramatically upon addition of the trans streptavidin, indicating that the biotin at residue 1 had reached the trans solution and could be grabbed by streptavidin, whereas in the case of LF_N 1C/32C/33C, the rate of decline remained unchanged, indicating that the biotin at residue 1 could not reach the trans solution. The dashed line is the zero current level. The results of experiments such as these are summarized in the inset, where a blue dot indicates that the biotin at residue 1 did not reach the trans solution, and a red dot indicates that the biotin at residue 1 had reached the trans solution. Also given in the inset is the length in angstroms of the distance from residue 1 to the biotin stopper for an α helix (α Å) and for an extended chain (ext Å). The cartoon is similar to that in Fig. 6 (see legend); X aa is the number of amino acids from residue 1 to the biotin stoppers.

channel ~ 170 Å (Fig. 1). Given that the N-terminal biotin of LF_N 1–43 was grabbed by cis streptavidin, and remembering that there are three amino acids linking LF_N to YFP, we come up with a distance of 162 Å (45×3.6 Å), which is close to 170 Å. This suggests that YFP does not partially unfold to any significant extent and somewhat obviates the concern raised in the preceding paragraph. This is supported by our finding that the N-terminal biotin of LF_N 1–33, whose extended length is 126 Å (35×3.6 Å), was not grabbed by cis streptavidin.

The conclusion that the first 33 residues of LF_N traverse the stem of the channel in an extended configuration was derived from our experiments in which LF_N was added to the cis side, where we found that these 33 residues were sufficient to span the distance from the Φ-clamp to the trans solution. Because the structure of the first 27 residues of LF_N is unresolved, it is possible that they are disordered in solution and simply remain so in their translocation through the channel. Our experiments in which LF_N-YFP was added to the trans side, however, demonstrate that the first 43 residues of LF_N (plus the three-amino acid linker) are sufficient to traverse the entire channel. Because the region from residue 28 to 43 is α helical in the crystal structure (Pannifer et al., 2001),

this finding means that this region unfolds under the conditions of our experiments and suggests that the (PA₆₃)₇ channel actually unfolds the secondary structure of LF_N as it is being translocated. Although this conclusion strictly applies only to the first 43 residues, there is no reason to believe that this is not equally true for any stretch of amino acids in the polypeptide chain.

Biotin as a stopper

All of our experiments depended on the fact that a biotin attached to the N terminus of LF_N readily traverses the (PA₆₃)₇ channel. On the other hand, we showed that a biotin attached at most other positions in LF_N was stopped at the Φ-clamp and therefore could not traverse the channel (Fig. 9). It was this fact that allowed us to use biotin (or two successive biotins) as a stopper. Why is the N-terminal biotin different from (almost) all other biotins? At first thought, one might attribute this difference as arising in some way from the fact that the N terminus is the first residue to pass through the Φ-clamp. This, however, cannot be the case because all of the experiments reported here were done with a 20-residue (His)₆ tag attached to the N terminus of LF_N. Thus, position 1 of LF_N, its N terminus, is as much an

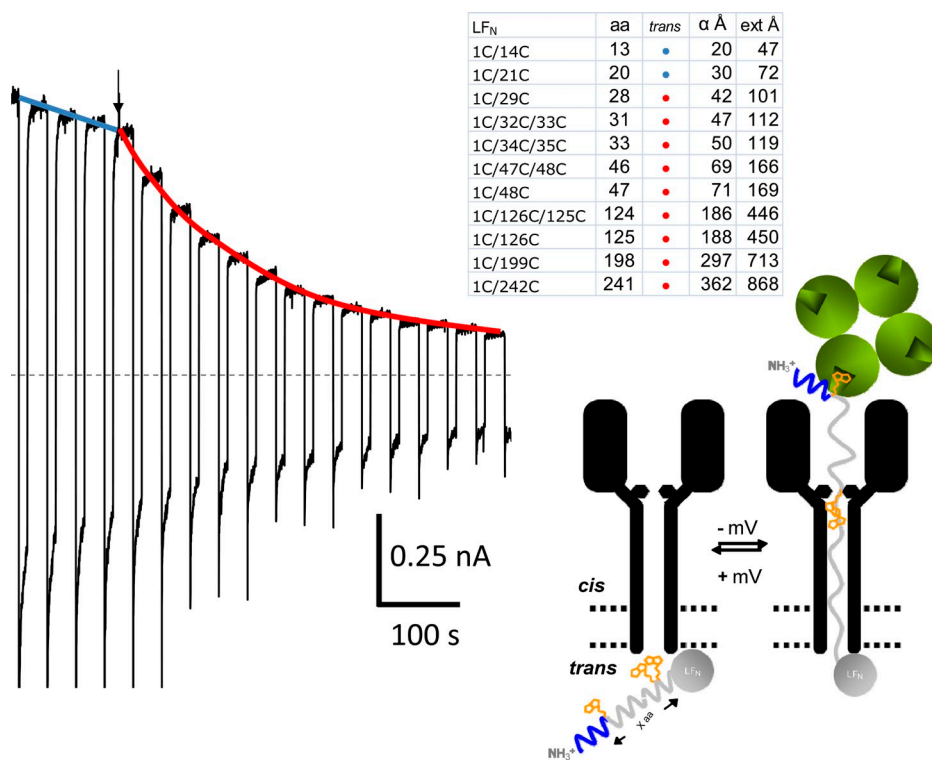


Figure 11. Demonstration that the (PA₆₃)₇ channel can translocate LF_N (N-terminal end first) from the trans to the cis solution. This is essentially the same type of experiment as the one depicted in Fig. 10, except in this case, the (His)₆-LF_N cysteine mutant (LF_N 1C/126C with these residues biotinylated) was added to the trans side and streptavidin was added to the cis side. After the (PA₆₃)₇-induced conductance had leveled off, the LF_N cysteine mutant was added to the trans side to a concentration of 120 nM, and the voltage protocol of -50 mV for 10 s and $+20$ mV for 33 s was begun. At the arrow, streptavidin was added to the cis compartment to a concentration of 1 μM. We see that before the addition of streptavidin, the level of unblocking was declining, having not yet reached a steady-state. (Note that because unblocking is occurring at a positive voltage [$+20$ mV] rather than at a negative voltage, it is not obscured by the intrinsic (PA₆₃)₇ gating and in fact occurs very rapidly.) The rate of decline, however, increased dramati-

cally upon the addition of cis streptavidin, indicating that the biotin at residue 1 had reached the cis solution and could be grabbed by streptavidin. The dashed line is the zero current level. The results of experiments such as this are summarized in the inset, where a blue dot indicates that the biotin at residue 1 did not reach the cis solution, and a red dot indicates that it did. Also given in the inset is the length in angstroms of the distance from residue 1 to the biotin stopper for an α helix (α Å) and for an extended chain (ext Å). The cartoon is virtually identical to the one in Fig. 10, except in this instance, LF_N has been added to the trans side and streptavidin was added to the cis side.

“interior” position as any other. Another possibility is that because there are two glycines immediately following position 1, there is room for some water molecules to associate with the biotin, and that somehow this facilitates passage of biotin through the Φ -clamp. That this may be the explanation is indicated by our observation that LF_N with a biotin attached to position 77, which is also immediately followed by two glycines, is translocated as readily as wild-type LF_N (unpublished data). Interestingly, a similar phenomenon to that seen with biotin is also seen with SO_3^- ; namely, LF_N with an SO_3^- introduced at either position 1 (the N terminus) or position 77 translocates as readily as wild-type LF_N , but with an SO_3^- at other positions it translocates very slowly (Basilio et al., 2009).

A puzzling difference between the YFP and biotin stopper experiments

In those experiments with YFP as the stopper, if trans streptavidin grabbed the N-terminal biotin, then ultimately

virtually all of the $(\text{PA}_{63})_7$ channels ended up blocked (e.g., Fig. 6). In contrast to this, in those experiments in which biotins acted as the stopper, if trans streptavidin grabbed the N-terminal biotin, only a fraction of $(\text{PA}_{63})_7$ channels were ultimately blocked (e.g., Fig. 10). One possible explanation for this is that with the N-terminal biotin held by streptavidin, the biotin stoppers are dragged through the Φ -clamp. Thus, a steady-state is reached between the rate at which streptavidin grabs the N-terminal biotin of LF_N and thereby keeps the channels blocked, and the rate at which LF_Ns with grabbed N-terminal biotins are translocated and thereby unblock the channels. We tested this hypothesis in a translocation experiment, such as the one depicted in red in Fig. 9 A, by adding streptavidin to the trans solution, but we saw no significant increase in the rate of translocation of LF_N (unpublished data). At this point, we have no explanation for why only a fraction of the channels was ultimately blocked.

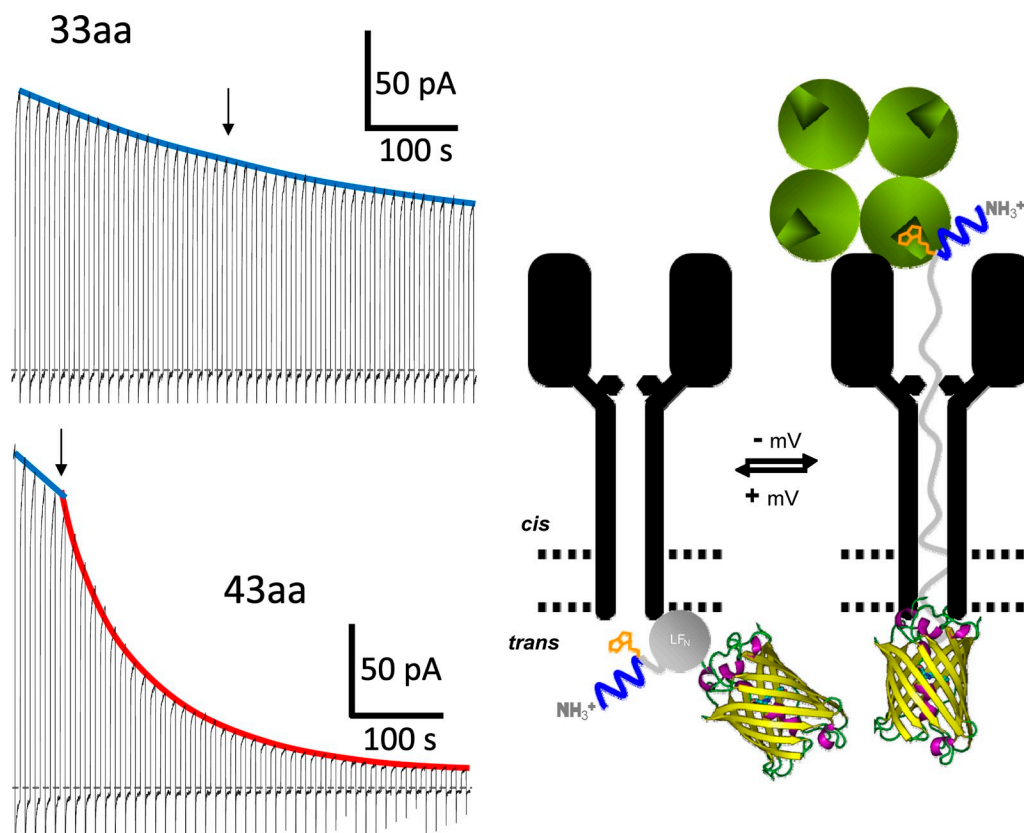


Figure 12. Example of backward translocation of a $(\text{His})_6\text{-LF}_\text{N}$ construct (LF_N 1–43 YFP with 1C biotinylated) whose N terminus reached the cis solution, and a $(\text{His})_6\text{-LF}_\text{N}$ construct (LF_N 1–33 YFP with 1C biotinylated) whose N terminus did not reach the cis solution. This is essentially the same type of experiment as the one depicted in Fig. 6, except in this case, the relevant LF_N -YFP mutant was added to the trans side and streptavidin was added to the cis side. After the $(\text{PA}_{63})_7$ -induced conductance had leveled off, the LF_N -YFP mutant was added to the trans side to a concentration of 240 nM, and the voltage protocol of -80 mV for 6 s and $+30$ mV for 5.5 s was begun. At the arrow, streptavidin was added to the cis compartment to a concentration of 1 μM . We see that before the addition of streptavidin, the level of unblocking was declining, having not yet reached a steady state. Most significantly, however, is that in the case of LF_N 1–43 YFP, this rate of decline increased dramatically upon the addition of the cis streptavidin, indicating that the biotin at residue 1 had reached the cis solution and could be grabbed by streptavidin, whereas in the case of LF_N 1–33 YFP, the rate of decline remained unchanged, indicating that the biotin at residue 1 could not reach the cis solution. The dashed line is the zero current level. The cartoon is virtually identical to that in Fig. 11, except that here YFP, instead of biotins, is the stopper.

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REFERENCES

- Anderson, D.S., and R.O. Blaustein. 2008. Preventing voltage-dependent gating of anthrax toxin channels using engineered disulfides. *J. Gen. Physiol.* 132:351–360. doi:10.1085/jgp.200809984
- Basilio, D., S.J. Juris, R.J. Collier, and A. Finkelstein. 2009. Evidence for a proton–protein symport mechanism in the anthrax toxin channel. *J. Gen. Physiol.* 133:307–314. doi:10.1085/jgp.200810170
- Blaustein, R.O., T.M. Koehler, R.J. Collier, and A. Finkelstein. 1989. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. *Proc. Natl. Acad. Sci. USA.* 86:2209–2213. doi:10.1073/pnas.86.7.2209
- Cunningham, K., D.B. Lacy, J. Mogridge, and R.J. Collier. 2002. Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. *Proc. Natl. Acad. Sci. USA.* 99:7049–7053. doi:10.1073/pnas.062160399
- Dickerson, R.E., and I. Geis. 1969. *The Structure and Action of Proteins*. Benjamin/Cummings Publishing Company, Menlo Park, CA. 120 pp.
- Feld, G.K., K.L. Thoren, A.F. Kintzer, H.J. Sterling, I.I. Tang, S.G. Greenberg, E.R. Williams, and B.A. Krantz. 2010. Structural basis for the unfolding of anthrax lethal factor by protective antigen oligomers. *Nat. Struct. Mol. Biol.* 17:1383–1390. doi:10.1038/nsmb.1923
- Finkelstein, A. 2009. Proton-coupled protein transport through the anthrax toxin channel. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364:209–215. doi:10.1098/rstb.2008.0126
- Katayama, H., B.E. Janowiak, M. Brzozowski, J. Juryck, S. Falke, E.P. Gogol, R.J. Collier, and M.T. Fisher. 2008. GroEL as a molecular scaffold for structural analysis of the anthrax toxin pore. *Nat. Struct. Mol. Biol.* 15:754–760. doi:10.1038/nsmb.1442
- Kintzer, A.F., K.L. Thoren, H.J. Sterling, K.C. Dong, G.K. Feld, I.I. Tang, T.T. Zhang, E.R. Williams, J.M. Berger, and B.A. Krantz. 2009. The protective antigen component of anthrax toxin forms functional octameric complexes. *J. Mol. Biol.* 392:614–629. doi:10.1016/j.jmb.2009.07.037
- Krantz, B.A., A.D. Trivedi, K. Cunningham, K.A. Christensen, and R.J. Collier. 2004. Acid-induced unfolding of the amino-terminal domains of the lethal and edema factors of anthrax toxin. *J. Mol. Biol.* 344:739–756. doi:10.1016/j.jmb.2004.09.067
- Krantz, B.A., R.A. Melnyk, S. Zhang, S.J. Juris, D.B. Lacy, Z. Wu, A. Finkelstein, and R.J. Collier. 2005. A phenylalanine clamp catalyzes protein translocation through the anthrax toxin pore. *Science*. 309:777–781. doi:10.1126/science.1113380
- Krantz, B.A., A. Finkelstein, and R.J. Collier. 2006. Protein translocation through the anthrax toxin transmembrane pore is driven by a proton gradient. *J. Mol. Biol.* 355:968–979. doi:10.1016/j.jmb.2005.11.030
- Lacy, D.B., M. Mourez, A. Fouassier, and R.J. Collier. 2002. Mapping the anthrax protective antigen binding site on the lethal and edema factors. *J. Biol. Chem.* 277:3006–3010. doi:10.1074/jbc.M109997200
- Lacy, D.B., D.J. Wigelsworth, R.A. Melnyk, S.C. Harrison, and R.J. Collier. 2004. Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. *Proc. Natl. Acad. Sci. USA.* 101:13147–13151. doi:10.1073/pnas.0405405101
- Melnyk, R.A., K.M. Hewitt, D.B. Lacy, H.C. Lin, C.R. Gessner, S. Li, V.L. Woods Jr., and R.J. Collier. 2006. Structural determinants for the binding of anthrax lethal factor to oligomeric protective antigen. *J. Biol. Chem.* 281:1630–1635. doi:10.1074/jbc.M511164200
- Mueller, P., D.O. Rudin, H.T. Tien, and W.C. Westcott. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* 67:534–535.
- Nguyen, T.L. 2004. Three-dimensional model of the pore form of anthrax protective antigen. Structure and biological implications. *J. Biomol. Struct. Dyn.* 22:253–265.
- Pannifer, A.D., T.Y. Wong, R. Schwarzenbacher, M. Renatus, C. Petosa, J. Bienkowska, D.B. Lacy, R.J. Collier, S. Park, S.H. Leppla, et al. 2001. Crystal structure of the anthrax lethal factor. *Nature*. 414:229–233. doi:10.1038/n35101998
- Perez-Jimenez, R., S. Garcia-Manyes, S.R. Aivarapu, and J.M. Fernandez. 2006. Mechanical unfolding pathways of the enhanced yellow fluorescent protein revealed by single molecule force spectroscopy. *J. Biol. Chem.* 281:40010–40014. doi:10.1074/jbc.M609890200
- Pimental, R.A., K.A. Christensen, B.A. Krantz, and R.J. Collier. 2004. Anthrax toxin complexes: heptameric protective antigen can bind lethal factor and edema factor simultaneously. *Biochem. Biophys. Res. Commun.* 322:258–262. doi:10.1016/j.bbrc.2004.07.105
- Qiu, X.-Q., K.S. Jakes, P.K. Kienker, A. Finkelstein, and S.L. Slatin. 1996. Major transmembrane movement associated with colicin Ia channel gating. *J. Gen. Physiol.* 107:313–328. doi:10.1085/jgp.107.3.313
- Rekas, A., J.R. Alattia, T. Nagai, A. Miyawaki, and M. Ikura. 2002. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. *J. Biol. Chem.* 277:50573–50578. doi:10.1074/jbc.M209524200
- Wonderlin, W.F., A. Finkel, and R.J. French. 1990. Optimizing planar lipid bilayer single-channel recordings for high resolution with rapid voltage steps. *Biophys. J.* 58:289–297. doi:10.1016/S0006-3495(90)82376-6
- Young, J.A., and R.J. Collier. 2007. Anthrax toxin: receptor binding, internalization, pore formation, and translocation. *Annu. Rev. Biochem.* 76:243–265.
- Zhang, S., A. Finkelstein, and R.J. Collier. 2004a. Evidence that translocation of anthrax toxin's lethal factor is initiated by entry of its N terminus into the protective antigen channel. *Proc. Natl. Acad. Sci. USA.* 101:16756–16761. doi:10.1073/pnas.0405754101
- Zhang, S., E. Udho, Z. Wu, R.J. Collier, and A. Finkelstein. 2004b. Protein translocation through anthrax toxin channels formed in planar lipid bilayers. *Biophys. J.* 87:3842–3849. doi:10.1529/biophysj.104.050864